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Award Number: DAMD17-97-1-7155

TITLE: Estrogens, Microtubules, and Aneuploidy: Mechanisms of
         Mammary Gland Tumorigenesis

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REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Final (15 Jun 1997 – 14 Jun 2002)

4. TITLE AND SUBTITLE
Estrogens, Microtubules, and Aneuploidy: Mechanisms of Mammary Gland Tumorigenesis

5. FUNDING NUMBERS
DAMD17-97-1-7155

6. AUTHOR(S)
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20040130 024

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
The role of estradiol (E2) metabolism in mammary carcinogenesis was studied in a rat model. Two strains were employed: the ACI is extremely sensitive to E2 carcinogenicity, whereas the Sprague-Dawley is resistant. Characterization of E2 metabolism in liver microsomal prepartions showed robust metabolism at substrate concentrations form $60 \phi M$ to 3nM. The latter value approximates the physiological concentration of E2. In the micromolar range both strains produce estrone and 2-hydroxy-E2. In the nanomolar range, however, estrone is the dominant product from the Sprague-Dawley whereas the ACI makes almost entirely 4-hydroxy-E2. Given that 4-hydroxy-E2 may be the key carcinogenic metabolites of E2, this strain-specific metabolism may explain the noted difference in E2 carcinogenicity between the two strains. Profiling of cytochrome P450 enzymes (CYPs) in the livers of the two strains by Western analysis did not show significant differences. Particularly noteworthy was the absence of CYP1B1, the purported estrogen 4-hydroxylase. Although liver determines the systemic fate of E2, metabolism and effects of E2 in target tissues are of even more concern. We tried to extend these studies into a rat mammary epithelial cell (RMEC) system. We isolated RMEC from both rat strains, but the result cultures were mixed population containing both myoepithelial and luminal epithelial cells. Minimal metabolic activity was seen, and the viability of these cultures was poor, preventing the continuation of these studies.

14. SUBJECT TERMS
Estradiol, catechol, CDA, mammary gland, liver

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Estrogen exposure represents one of the few clear risk factors for breast cancer in humans. This potential risk is underscored by the finding that both natural and synthetic estrogens are mammary carcinogens in animal models. Despite this clear association between estrogens and breast cancer, the mechanism of tumorigenesis by these agents has not been established. This project was designed to test the hypothesis that estrogens or their metabolites, via covalent binding, are specific disruptors of mitotic microtubules in mammary epithelial cells, and that the resulting interference with mitosis causes aneuploidy. To test this hypothesis, we planned to study the metabolism and effects of the mammary carcinogen estradiol (E2), in liver microsomes and in rat mammary epithelial cell lines (RMEC). Microsomes and mammary epithelial cells were isolated from female ACI, Sprague-Dawley, and Copenhagen (COP) rats. The former strain is extremely sensitive to estrogens as mammary carcinogens, whereas the Sprague-Dawley and COP strains are relatively resistant to estrogens. The concentration- and time-dependence of metabolism and the covalent modification of proteins was to be determined for E2 in preparations from each rat strain, as was the specific covalent modification of tubulin subunits. These chemical endpoints were to be compared with the determinations of several cellular changes in response to each of the compounds. Effects on cell proliferation were to be assessed by immunohistochemical analysis of the proliferation-specific nuclear antigen PCNA (19A2). Effects on the integrity of microtubules (mitotic spindles) also were to be determined immunohistochemically using anti-rat tubulin antibodies. Finally, aneuploidy was to be analyzed by flow cytometry. These studies of the effects of the compounds on subcellular structure and function constitute a step-wise examination of the proposed mechanism of action for carcinogenic estrogens. Induction of proliferation would be used as a general index of estrogenicity. The effects on microtubules, however, would begin to explore the link between covalent modification of tubulin in a target cell for carcinogenicity of these compounds and the disruption of mitosis. If the covalent modification of tubulin, the disruption of microtubules, and the induction of aneuploidy followed the same time- and concentration-dependence then a strong mechanistic link would be forged. This would support future studies on the induction of aneuploidy by estrogens in mammary epithelial cells *in vivo*.

Body

E2 metabolism by RLM-- The findings reported in previous annual reports for this project were confirmed and expanded. These are described in detail in the accompanying publications (Appendix) Briefly, RLM from both female ACI and SD rats actively carry out the NADPH-dependent oxidation of E2. All incubations included RLM, radiolabeled E2, and a glucose-6-phosphate dehydrogenase-based NADPH generating system. Reactions were initiated by the addition of glucose-6-phosphate, and were run at 37° C. Reactions were terminated and radiolabeled compounds extracted by the addition of 5 volumes of ethyl acetate. Extracted materials were analyzed by reverse phase HPLC with radiometric detection. At starting E2 concentrations above 1 µM the predominant product is estrone (E1), with lesser amounts of 2-hydroxy-E2 formed. As the substrate concentration is decreased, however, quantitative and qualitative changes occur in these product profiles. The SD microsomes show a progressive shift towards 2-hydroxylation, with diminishing relative amounts of E1 being produced. This fits with 17 - hydroxysteroid dehydrogenase being a lower affinity enzyme for E2 than are the CYPs involved in hydroxylation reactions. An even more striking change occurs in the incubations containing ACI RLM. Not only does aromatic hydroxylation become the dominant pathway, but moreover a profound shift from only 2-hydroxylation at high E2 concentration to primarily 4-hydroxylation is seen at nanomolar concentrations of E2. This shift in product profile suggests that a different CYP is present in the ACI rat

liver than in the SD preparation. Most CYPs that hydroxylate the A ring of E2 greatly favor attack at the 2-position, rather than at the 4-position. The exception is the rat isozyme CYP 1B1 (1). It is noteworthy that the K_m determined by us for the 4-hydroxylation of E2 in ACI RLM, 0.8 ± 0.3 µM, compare favorably with the value of 0.71 µM reported by Sutter *et al.* (1) for 4-hydroxylation of E2 by recombinant rat CYP 1B1. We examined the specific CYP content of ACI and SD RLM by performing Western immunoblot analysis.

Analysis for CYP 1A1 and 1A2 was negative for both the ACI and the SD RLM . Antibodies against CYP 2B1/2, however, showed that this CYP was expressed in both strains. Expression levels were noticeably higher in the ACI than in the SD rats. Treatment with s.c. E2 pellets caused some decrease in the levels of CYP 2B detected, but this may not be a significant difference. The highest level of constitutive expression was for CYP 3A1/2. E2 treatment did not affect the expression levels. The microsomes from 6 week old rats showed higher 3A levels in the ACI than in the SD rats, but this difference was not apparent in RLM from 12 week old animals. None of these differences correlated with the marked change in product profile between the ACI and the SD rat.

It has become generally accepted that CYP 1B1 is the specific estradiol 4-hydroxylase. It is also assumed that CYP 1B1 is not constitutively expressed in rat liver. In collaboration with Nigel Walker at NIEHS, we analyzed our RLM by Western immunoblotting for CYP 1B1. That protein was not detectable in microsomes from either rat strain. The summation of our immunoblotting for CYPs is that there are no significant differences in the expression levels of any of these CYPs between the two strains, and thus no clear enzymological basis for the profound differences in hydroxylation pattern for E2 has been found. We are left with two hypotheses, neither of which will be tested within the current project. The first hypothesis is that a different CYP than those analyzed by our immunoblotting is present in the ACI RLM, and that this as yet unidentified enzyme is the specific high affinity, low capacity 4-hydroxylase responsible for the observed product profile for E2. The second hypothesis is that there is a polymorphism in one of the known CYPs in the ACI rat, and that this polymorphism results in an altered binding orientation for E2 in the active site of the enzyme. This altered binding orientation results in the 4-position of the A ring of the substrate being available for hydroxylation, rather than the 2-position as in the major form of the enzyme.

RMEC culture-- We have shown previously that liver microsomal preparations from female ACI and Sprague-Dawley rats produce dramatically different oxidative metabolites of E2 (4). Specifically, at low E2 concentrations, ACI rat liver produces primarily 4-hydroxy-E2, the presumed carcinogenic metabolite of E2. Sprague-Dawley liver preparations, under the same conditions, produce little if any detectable 4-hydroxy-E2 and instead produce 2-hydroxy-E2, a non-carcinogenic metabolite. Thus the specific products formed by liver microsomes from the two strains fall in line with their relative susceptibilities to E2-induced mammary tumors-- the sensitive ACI strain produces the active carcinogen while the resistant Sprague-Dawley strain produces the non-carcinogenic product. The goal of the project was to move on to the characterization of target-tissue metabolism of E2 by the two strains, using primary and secondary cultures of mammary epithelial cells. This step has been unsuccessful. We have isolated mammary epithelial cells from young female ACI and Sprague-Dawley rats on eight occasions. These cells have been cultured in 100 mm dishes coated with rat tail collagen, using DMEM/F12 medium with 10% serum. These conditions are used routinely in this institution for the culture of mouse mammary epithelial cells (2).

Our preparations of RMEC resulting from collagenase digestion and enrichment by Percoll gradient centrifugation yielded myoepithelial cells as the dominant cell type. Although there may be some advantages to having a mix of cells similar to that occurring *in situ*, we had a major concern about this mixed population. First, myoepithelial cells do not appear to be target cells for tumor development. Second, it appears that our culture conditions with DMEM/F12 medium and 10% porcine serum select for the preferential growth of the myoepithelial cells at the expense of the lumenal and alveolar epithelial cells. We reviewed the literature and attempted to test chemically-defined medium developed for the culture of RMEC by Ip and coworkers (2,3). We tried to measure the basal and E2-dependent effects on growth rate of the cells, and on the relative distribution of myoepithelial and lumenal epithelial cells. Overall cell proliferation was to be measured using a standard MTT tetrazolium salt reduction assay. Myoepithelial cells would be detected and counted as actin-positive cells by immunofluorescence, and lumenal and alveolar cells quantified similarly using peanut agglutinin as a specific probe. The goal was to have a complete characterization of the distribution of cell types within our RMEC preparations, and more importantly a determination of the effects of E2 on these distributions. Unfortunately, we were unable to achieve sufficient viability and growth of RMEC to support our studies. We tried eight separate fresh cell preparations, and three separate trials with cryopreserved cells. Although 100 mm plates were seeded with 10^6 cells, and initial growth was observed, the plates never exceeded about 60% confluence, and by that time many dead and dying cells were observed. Without this stable cell population the proposed studies of E2 metabolism and effects could not be performed. Our consultation with others who routinely cultured either art or mouse MEC did not isolate any clear deficiency in our procedures.

A related model system was chosen to replace primary cultures of RMEC for the remainder of the funding period. The laboratory of Jim Shull at the Eppley Cancer Center in Omaha has isolated and characterized spontaneously immortalized non-transformed epithelial cell lines from the mammary glands of the ACI rat and from its' E2-resistant parental strain, the Copenhagen rat (COP). These cells were reported to grow readily in low- or no-serum media and retain much of their epithelial morphology and behavior. We proposed to use this established, robust cell system for our remaining studies.

The use of the mammary epithelial cell lines would retain the essential feature of the original experimental design, namely to study estrogens in target cell populations from strains sensitive to and resistant to the carcinogenic effects of estrogens. In fact, using RMEC derived from the Copenhagen rat, an estrogen-resistant parental strain for the ACI hybrid, would provide an arguably closer match between the two strains with the exception of the marked difference in response to estrogens, and thus a cleaner system for sorting out key mechanistic steps related to carcinogenesis. The use of the cell lines also clearly would bypass our failure to produce viable early passage cultures of these cells. The major drawback to the use of these cell lines is the assumption that, like virtually all epithelial cells, they will have ceased expression of cytochrome P450s after so many passages. We intended to verify this by examination of E2 metabolism, but regardless of the result we would press ahead with investigations of how cells from the two strains respond to E2 and its metabolites.

Samples of the ACI and COP RMEC were obtained from the Shull laboratory. Unfortunately, these studies were not initiated. Although the time period of this award was extended for an additional year, no additional funds were available from either internal or external sources. Indeed, the scope of this project was far greater than could have been supported by the $10,000 annually for 4 years provided by my institution. The success of this project relied on the ability to obtain additional funding in the form of an NIH grant or similar scale funding. Despite attempts to obtain such funding, no additional awards were

made. This lack of financial support seriously compromised the completion of the specific aims to be performed in cell culture.

A second impediment to the completion of this project was the decrease in faculty size experienced by the department of the Principal Investigator. With the faculty roster dropping from 16 in 1997 to 10 in 2000, the resultant increase in teaching and administrative duties of remaining faculty was substantially increased.

A final problem was the lack of support from the Hormonal Carcinogenesis Laboratory at the University of Kansas Medical Center. Although some facilities and equipment were provided for the attempts at preparation of RMEC, overall assistance and interest in this project was absent. Not only did the Director of the laboratory fail to guide my traineeship as was promised in support of my initial application, but he entered into collaboration with researchers at another institution which resulted in the publication which directly challenged our major findings (5). His "guidance" consisted of telling me about this challenge to our findings not when different results were obtained, but rather after the manuscript had been submitted for publication. This total lack of support severely impacted the necessary acquisition of new skills and of reagents required for the proposed studies in cell culture.

Key Research Accomplishments

X   Demonstration of 4-hydroxy-E2 as major metabolite of E2 in ACI rat liver

X   Determined kinetic constants for 2- and 4-hydroxylation of E2 in ACI and SD rat liver preparations

X   Determined that CYP 1B1 is _not_ the E2 4-hydroxylase in the ACI rat liver

Conclusions

Despite a recent report challenging our findings, we stand behind our observation that RLM from ACI rats efficiently convert E2 to 4-hydroxy-E2 when initial substrate concentrations are in the nanomolar range. Moreover, this 4-hydroxylation is not catalyzed by CYP1B1, the only known estrogen 4-hydroxylase. This suggests that either a different CYP than those normally associated with the formation of catechol estrogens is involved, or that a polymorphism in one of the typical catechol-forming CYPs alters the regioselectivity of the hydroxylation reaction. Given the evidence supporting 4-hydroxy-estrogens as more carcinogenic metabolites of E1 and E2, then increased formation of these metabolites could well be a contributing factor in breast carcinogenesis. Determining the identity of this 4-hydroxylase, and searching for a human analogue, would provide important tools for determining the role of this metabolic step in estrogen carcinogenicity. If this pathway was found to be important in estrogen carcinogenicity in breast, then screening for this CYP phenotype or genotype might be an important factor in assessment of overall risk of breast cancer.

Reportable Outcomes

Publications

Abstracts

Reed, G.A., Wilson, A.M., and Hayden, J.K.: Estradiol metabolism in rat strains differing in susceptibility to mammary carcinogenesis. *Third International Symposium on Hormonal Carcinogenesis*, 1998.

Wilson, A.M., Padgitt, J.K., and Reed, G.A.: Estradiol metabolism in rat strains differing in susceptibility to mammary carcinogenesis. *Toxicological Sciences* **1-S**: 238, 1999.

Reed, G.A. and Wilson, A.M.: Estradiol metabolism in rat strains differing in susceptibility to mammary carcinogenesis. *Proceedings, Era of Hope Department of Defense Breast Cancer Research Program Meeting, Vol. II*, 467, 2000.

Papers

Reed, G.A., Wilson, A.M., and Padgitt, J.K.: Estradiol metabolism by rat liver microsomes from strains differing in susceptibility to mammary carcinogenesis. In: *Hormonal Carcinogenesis III* (J.J. Li, J.R. Daling, S.A. Li, eds.), pp. 451-455, Springer-Verlag, New York, 2001.

Wilson, A.M. and Reed, G.A.: Predominant 4-hydroxylation of estradiol by constitutive cytochrome P450s in the ACI rat liver. *Carcinogenesis* **22**: 257-263, 2001.

Funding Application Based on this Work

NIH/NCI, "Estrogens, Aneuploidy, and Breast Cancer", Submitted 10/01/98
$507,061 total direct costs. Not funded.

Degree Awarded

Wilson, Angela M., M.S. in Pharmacology, Department of Pharmacology, Toxicology, and Therapeutics, School of Medicine, University of Kansas. Defended August 31, 2000.

Personnel Funded form This Award

Reed, Gregory A.

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Estradiol Metabolism by Rat Liver Microsomes from Strains Differing in Susceptibility to Mammary Carcinogenesis

Gregory A. Reed, Angela M. Wilson, and Janette K. Padgitt

Summary

Rat strains are known to differ markedly in their susceptibility to estrogen (E)-induced mammary tumors. Specifically, the ACI strain is extremely sensitive to Es, whereas the Sprague-Dawley (SD) strain is relatively resistant. We have compared the metabolism of estradiol (E_2) by liver microsomes from these two rat strains. Both strains exhibit hydroxysteroid dehydrogenase activity, with estrone (E_1) being the major product at E_2 concentrations above 1 μM. Some A-ring hydroxylation also is seen. As the E_2 concentration is decreased, however, hydroxylation becomes a more dominant pathway for both strains. In the SD preparations this still yields E_1 as the major product. ACI liver, however, produces primarily hydroxylated-E_2 at these concentrations. This difference is most apparent at E_2 concentrations below 100 nM. This difference in the disposition of E_2 and its being most pronounced at E_2 concentrations nearing the physiological range, suggest that this difference may contribute to the relative sensitivity of these strains to E_2 carcinogenicity.

Introduction

The identification of carcinogenic risks may be derived from two complementary approaches: the first is epidemiological analysis of human populations, while the second is by extrapolation from controlled exposure studies with experimental animals. The association between Es and breast cancer has been supported by ample data from both approaches. Despite the clear association between Es and breast cancer, the mechanisms involved in this effect are not clear. A pronounced strain difference in sensitivity to mammary carcinogenesis by Es provides an opportunity to identify key mechanistic features of the process. The

ACI rat is sensitive to Es as mammary carcinogens (1-4). Sprague-Dawley rats, in contrast, are relatively resistant to E carcinogenicity (2,5). By comparing E metabolism and resultant effects in the two strains it not only is possible to look for differences in metabolism and responses, but also to try and correlate these responses with susceptibility to carcinogenesis. The examination of estradiol (E_2) metabolism by liver microsomal preparations from these two strains represents the first step in these comparative studies.

Materials and Methods

Female Sprague-Dawley and ACI rats, 6 to 7 weeks old, were purchased from Harlan Laboratories. The livers were homogenized and microsomes prepared by differential centrifugation. Microsomal pellets were resuspended in phosphate-$MgCl_2$ buffer, pH 7.4, and stored at -80° C.

[^{3}H]-E_2 was incubated with RLM (1 mg protein/ml) in 50mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl2, 5 mM glucose-6-phosphate, and 1 mM ascorbate at 37° C. The NADPH generating system, where noted, included 1 U/ml glucose-6-phosphate dehydrogenase and was initiated by the addition of 1 mM NADP. Reactions with NADP or NAD as cofactors were initiated by the addition of these compounds, again to a final concentration of 1 mM. Incubations were continued for 10 to 60 min, as indicated, depending on substrate concentration. The reaction was stopped by the rapid extraction with 2 x 3 vol of ethyl acetate. The combined extracts were evaporated under vacuum, and the residue dissolved in the initial acetonitrile-methanol-water-acetic acid mix of the HPLC elution.

Separation of metabolites was performed on a Supelcosil C18 column (5 μ, 4.6 mm x 25 cm) at 30° C using a gradient of acetonitrile, methanol, water, and 0.1% acetic acid (6). Unlabeled metabolite standards were detected by monitoring absorbance at 280 nm, whereas labeled metabolites were detected by a Packard Flo-One Beta detector with Ultima Flo M liquid scintillant.

Results

Liver microsomal preparations from female ACI and SD rats both catalyze extensive NADPH-dependent metabolism of E_2. Oxidation of E_2 to E_1 and aromatic hydroxylation of E_2 were the only products observed with these preparations. Maximal rates of E1 formation exceed 300 pmol mg^{-1} min^{-1} in both strains, whereas maximal rates of aromatic hydroxylation are greater than 6 pmol mg^{-1} min^{-1} in SD microsomes and 30 pmol mg^{-1} min^{-1} in ACI liver microsomes. Extensive metabolism was observed not only at the commonly used micromolar concentrations of E_2 (7-10), but at substrate concentrations as low as 3 nM.

Although the metabolite profiles derived from the two strains are nearly identical at high substrate concentrations, a pronounced divergence emerges as the E_2 concentration drops below 1 µM (Figure 1). For both strains, aromatic hydroxylation becomes more pronounced as the E_2 concentration is decreased, but this shift is far more extensive in the ACI rat. As shown, at 100 nM E_2 aromatic hydroxylation to yield catechol Es becomes the dominant pathway for E_2 oxidation, comprising 73% of total metabolism. In the SD rat preparation, however, the fraction of metabolism resulting from aromatic hydroxylation only reaches 36% of the total. An additional 16% of the metabolites produced by the SD rat are catechol E_1 derivatives, which have undergone both 17β-dehydrogenation but also aromatic hydroxylation. Even combining the E_1 and E_2 catechol metabolites still only comprises 52% of total metabolism, as compared to the 73% catechols formed by the ACI liver microsomes under these same conditions.
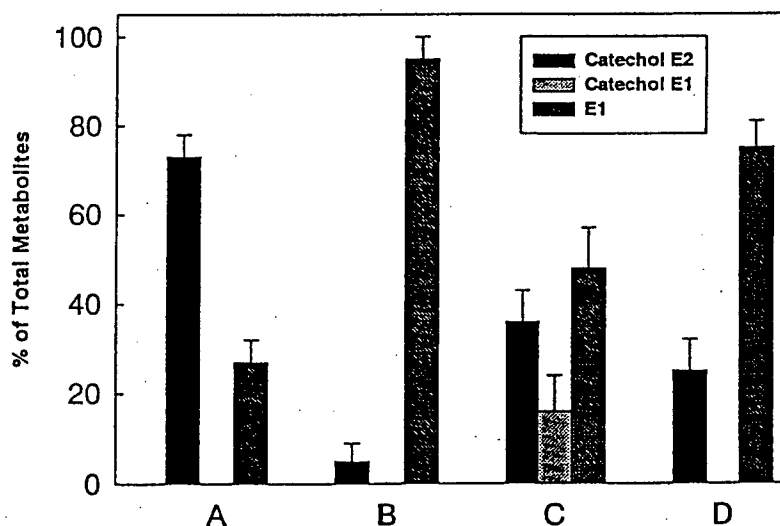

Figure 1. Product Distribution from E2 Oxidation by ACI and SD Rat Liver Microsomes: Effect of Substrate Concentration. Indicated concentrations of [^{3}H]-E_2 were incubated with 1 mg/ml microsomal protein with an NADPH generating system for 20 minutes. Extraction and analysis were as described. All data represent mean ± standard deviation from triplicate incubations. A, ACI rat, 100 nM E_2; B, ACI rat, 30 µM E_2; C, SD rat, 100 nM E_2; D, SD rat, 30 µM E_2.

The formation of hydroxylated products is assumed to be a cytochrome P450-dependent reaction, and thus NADPH-dependent, whereas the conversion to E_1 is a dehydrogenation which may utilize either NAD or NADP as a cofactor. This is borne out by the cofactor specificity demonstrated in Table 1. With the active glucose-6-phosphate dehydrogenase generating system maintaining most NADPH in the reduced form, fully 75% of E_2 metabolism is by hydroxylation, rather than dehydrogenation. The use of NADP, without the generating system

for reduction of the cofactor, results in a 3.5-fold increase in the rate of dehydrogenation to E_1, and a 70% decrease in aromatic hydroxylation. The residual hydroxylation presumably results from reduction of NADP via the 17β-hydroxysteroid dehydrogenase reaction. Finally, when NAD is added as the cofactor, which is fully active as a 17β-hydroxysteroid dehydrogenase cofactor but inactive as a cytochrome P450 cofactor in either its oxidized or reduced form, no hydroxylation is observed and 100% of the E_2 is converted to E_1. These cofactor studies clearly support the assignment of E_1 formation to 17β-hydroxysteroid dehydrogenase and the aromatic hydroxylation as a cytochrome P450-dependent reaction.

Table 1. Cofactor effect on E_2 oxidation by ACI rat liver microsomes.

Cofactor	Aromatic Hydroxylation	Dehydrogenation
NADPH generating system	25.8 ± 1.1 pmol mg^{-1} min^{-1}	8.7 ± 2.2 pmol mg^{-1} min^{-1}
NADP	6.3 ± 0.8	20.5 ± 0.5
NAD	0	50 ± 0

1 μM E_2 was incubated for 20 minutes with 1 mg/ml microsomal protein and either the NADPH generating system or with 1 mM NADP or NAD. Extraction and analysis were as described in Materials and Methods. Results are means $\pm$ standard deviation from triplicate incubations.

Conclusions

1. Rat liver microsomes efficiently oxidize E_2 at concentrations as low as 3 nM.
2. 17β-Hydroxysteroid dehydrogenase is a relatively low affinity but high capacity system for E_2 oxidation in RLM. Cytochrome P450-dependent aromatic hydroxylation of E_2 is a relatively high affinity but low capacity system for E_2 oxidation in RLM.
3. RLM from the female ACI rat differ from those from other rat strains in that aromatic hydroxylation of E_2 is more dominant than it is in the SD rat.

Acknowledgment

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Predominant 4-hydroxylation of estradiol by constitutive cytochrome P450s in the female ACI rat liver

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The ACI rat is extremely sensitive to estrogens as mammary carcinogens, whereas the Sprague–Dawley strain is relatively resistant. Comparison of the disposition and effects of estrogens in these two strains should provide insights into the mechanisms of estrogen carcinogenicity. We have begun this investigation by comparing the metabolism of [^{3}H]17β-estradiol (E2) by liver microsomes prepared from female rats from each strain. Both strains produce estrone (E1) as the major product at E2 concentrations >1 μM, with smaller amounts of 2-hydroxy-E2 formed. As the E2 concentration is decreased, however, aromatic hydroxylation becomes a more dominant pathway for both strains. At starting E2 concentrations as low as 3 nM, Sprague–Dawley liver microsomes produced comparable yields of 2-hydroxy-E2 and E1. In contrast, ACI liver microsomes yielded a profound shift to aromatic hydroxylation as the dominant pathway as E2 concentrations dropped below 1 μM, and this shift reflected the production of 4-hydroxy-E2 as the predominant product. The apparent K_m for 4-hydroxylation of E2 is <0.8 μM, as opposed to ~4 μM for 2-hydroxylation, suggesting that different cytochrome P450s (CYPs) are responsible. Western immunoblotting of the liver microsomal preparations from ACI and Sprague–Dawley rats for CYPs known to catalyze 2- and 4-hydroxylation of E2 revealed that both strains contained comparable amounts of CYP 2B1/2 and 3A1/2, but no detectable amounts of CYP 1B1, the proposed E2 4-hydroxylase. Although this enzyme is not a constitutive CYP in Sprague–Dawley rat liver, its presence in ACI liver could provide a ready explanation for the predominance of 4-hydroxy-E2 as a product. The identity of the estradiol 4-hydroxylase in ACI rat liver and the role of this unique reaction in the heightened sensitivity to E2 carcinogenicity remain to be elucidated.

Introduction

Estrogens are risk factors for breast cancer in humans. Epidemiological evidence indicates that an increase in level or duration of exposure to 17β-estradiol (E2) and estrone (E1)

is associated with an increased incidence of breast cancer (1,2). Early menarche and late menopause are risk factors which increase duration of exposure to endogenous estrogens (3). Interruption of normal estrogen levels during pregnancy decreases estrogen exposure, thus late first full-term pregnancy and nulliparity increase a woman's chance of developing breast cancer (2,4–7). In addition, long-term use of hormone replacement therapies increases breast cancer risk due to an increase in the level of exposure and the duration of exposure to estrogens (1,7).

Animal studies provide more direct and quantifiable evidence for a causal role of estrogens in tumorigenesis (reviewed in refs 8,9). E1, E2 and the stilbene estrogen diethylstilbestrol induce mammary gland tumors in both rats and mice and estriol (E3) is also a mammary carcinogen in mice. The synthetic steroids norethynodrel and mestranol are mammary carcinogens in rats and dogs, respectively. In addition to these effects in mammary gland, estrogens are also carcinogenic in the liver, kidney, pituitary and various organs of the genitourinary tract of several species (10–12). Induction of tumors in multiple tissues and species strongly supports the IARC classification of estrogens as human carcinogens (13).

Although estrogens are known mammary carcinogens in animals and humans, the mechanisms of carcinogenesis are not clear. One proposed mechanism is based on the mitogenic action of estrogens in hormone-specific tissues (i.e. uterus and breast) mediated via estrogen receptors (2,8,14). With longer periods of enhanced proliferation there is an increased chance of mutations. Another mechanism has been proposed in which estrogens act as direct genotoxic carcinogens (15). Estrogen metabolites are able to covalently bind to DNA and the resulting modified sites in DNA are proposed to be pre-mutagenic lesions (11,16). The third proposed mechanism of E2 carcinogenicity is that estrogens or their metabolites are indirectly genotoxic. Reactive oxygen species, generated by redox cycling of estrogen metabolites, such as the catechols and hydroquinones, are suggested to be DNA damaging. This is thought to occur by oxidation or hydroxylation of the DNA to produce pre-mutagenic lesions which can lead to mutations (17,18). Finally, an epigenetic mechanism has been proposed based on the induction of aneuploidy which is concurrent with the emergence of cell transformation (19–21).

Determination of the primary mechanism of estrogen carcinogenicity will require an appropriate model system. The rat provides an excellent model for mammary carcinogenesis based on a low spontaneous tumor incidence and efficient induction of tumors by chemical agents (8,9). In addition, chemically induced rat mammary tumors clearly resemble human breast tumors based on their similar morphology and on the high degree of estrogen dependence seen in both rat and human tumors (reviewed in ref. 9). What is even more striking is the pronounced strain difference in rat sensitivities to mammary carcinogenicity. ACI rats are extremely sensitive to estrogens as mammary carcinogens, developing palpable

tumors after 3 months treatment with E2 and 100% mammary tumor incidence after 6 months treatment (10,22). Sprague–Dawley rats, however, are much less sensitive to estrogens, exhibiting no mammary tumors after 7 months treatment (23,24). This strain difference in susceptibilities to mammary carcinogenicity is apparently specific for estrogens as causative agents. ACI rats are less sensitive to induction of mammary tumors by 7,12-dimethylbenz[a]anthracene (DMBA), a known mammary carcinogen, than are Sprague–Dawley rats. In fact, 100% of Sprague–Dawley rats treated with DMBA develop mammary tumors within a few weeks (25), whereas, only 30% of ACI rats develop mammary tumors after 8.5 months treatment. ACI rats are relatively insensitive to other genotoxic mammary carcinogens as well (26,27). This unique strain difference, in which the ACI rat is specifically more sensitive to estrogens as mammary carcinogens, presents an invaluable tool for determining the key mechanistic steps of E2 carcinogenicity.

Sprague–Dawley rats and ACI rats differ markedly in their responses to E2 with regard to mammary tumorigenesis. These differences could be due to differences in E2 pharmacodynamics, pharmacokinetics or both. By careful comparison of the interactions between E2 and the cells and tissues from these two strains we may dissect out critical processes which underlie the pronounced difference in susceptibility to tumor induction. These critical differences may also illuminate the major mechanism involved in estrogen carcinogenicity in the mammary gland. We have begun by comparing the metabolism of E2 by liver microsomes from the two strains. Although hepatic metabolism may not play as central a role in the mammary effects of E2 as target tissue metabolism, we nonetheless consider the characterization of hepatic E2 metabolism to be a logical first step in comparing these strains and their overall response to E2. We have observed quantitative and qualitative differences in E2 metabolism in ACI and Sprague–Dawley liver microsomes. The differences in hepatic metabolism of E2 between the two strains of rats may be the first step in elucidating the key differences affecting susceptibility to mammary carcinogenesis.

Materials and methods

Animals and chemicals

Sexually immature (6–7-week-old) and sexually mature (12–14-week-old) female Sprague–Dawley and ACI rats were purchased from Harlan Laboratories (Indianapolis, IN). [2,4,6,7-³H]E2 (72 Ci/mmol) and [4-¹⁴C]E2 (54.1 mCi/mmol) were products of New England Nuclear (Boston, MA). E2, E1, 2-hydroxyestradiol (2-OH-E2), 4-hydroxyestradiol (4-OH-E2), E3, 2-hydroxyestrone (2-OH-E1), 4-hydroxyestrone (4-OH-E1), 6α-hydroxyestradiol (6α-OH-E2), clotrimazole and all other substrates, enzymes and cofactors were from Sigma Chemical Co. (St Louis, MO). All solvents and reagent chemicals were of HPLC grade and were purchased from Fisher Scientific (St Louis, MO). Scintillant fluid was Ultima Flo M from Packard Instrument Co. (Meriden, CT). NuPage gels and reagents used in western immunoblot analysis were purchased from Novex (San Diego, CA). The primary antibodies for CYPs 2B1/2 and 3A1/2 were provided by Xenotech LLC (Kansas City, KS), whereas rabbit anti-CYP 1B1 (28,29) was generously supplied by Thomas Sutter (Johns Hopkins University, Baltimore, MD). The secondary antibody utilized for visualization of CYPs 2B1/2 and 3A1/2 was alkaline phosphatase-conjugated anti-rabbit IgG and was purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). The BCIP/NBT phosphatase substrate was obtained from Kirkegaard Perry Laboratories (Gaithersburg, MD) and the PVDF membrane was from Millipore (Bedford, MA). Bound antibody against CYP 1B1 was detected with a horseradish peroxidase-conjugated donkey anti-rabbit IgG using the enhanced chemiluminescence method (Amersham Corp., Arlington Heights, IL).

Microsomal preparation

Where indicated, rats were treated with a s.c. cholesterol pellet with or without E2 (20 mg/pellet) for 1 week. Rats were anesthetized with CO_2, then killed by decapitation. The livers from 4–8 rats were pooled and homogenized in Tris–KCl buffer, pH 7.4, at 4°C. The homogenate was fractionated by differential centrifugation and the microsomal pellets were resuspended in phosphate/MgCl₂ buffer, pH 7.4, and stored at –80°C. Protein concentrations of the resulting rat liver microsomes (RLM) were determined using the Bradford reagent with bovine serum albumin as the standard.

Microsomal metabolism and analysis

[³H]E2 (0.1 µCi/incubation) or [¹⁴C]E2 (0.05 µCi/incubation) was mixed with unlabeled E2 and incubated with RLM in 50 mM Tris–HCl buffer, pH 7.4, containing 5 mM MgCl₂, 5 mM glucose 6-phosphate and 1 mM ascorbate at 37°C in a shaking water bath. E2 was added as a stock solution in DMSO, with a constant final concentration of 2% DMSO (v/v). The NADPH generating system, where used, included 1 U/ml glucose 6-phosphate dehydrogenase. Reactions were initiated by the addition of 1 mM NADP. Reactions with NADP and NAD as cofactors were initiated by addition of these compounds, to a final concentration of 1 mM, in the absence of glucose 6-phosphate dehydrogenase. Incubations were continued for 20 or 60 min as indicated, depending on substrate concentration. The reaction was stopped by extraction with 2×3 vol ethyl acetate. Recoveries of labeled compounds by extraction were routinely >99%. The combined extracts were evaporated under vacuum and the residue dissolved in the initial acetonitrile/methanol/water/acetic acid mix for HPLC elution. Unlabeled estrogen metabolite standards were added to verify retention times. All incubations were performed in triplicate and data are presented as means ± SD of three replicates.

HPLC analysis

Analysis of E2 metabolites was performed on a Supelcosil C18 column (5 µm, 4.6 mm×25 cm) at 30°C (Supelco, Bellefonte, PA). The elution conditions were based on a system developed by Robert Breuggemeier (personal communication). A binary solvent gradient consisting of acetonitrile/methanol/water/acetic acid was used for elution of the compounds. Specifically, solvent A was 21% acetonitrile/22% methanol/57% water/0.1% acetic acid and solvent B was 40% acetonitrile/60% water/0.33% acetic acid. The gradient was as follows: 0–15 min 100% solvent A; a linear increase from 15–25 min to 19% solvent B; a linear increase from 25–35 min to 20% solvent B; a linear increase from 31–53 min to 100% solvent B; the column was returned to initial conditions over 20 min. Retention times for unlabeled metabolite standards, as detected by monitoring absorbance at 280 nm, were as follows: 6α-OH-E2, 6.00 min; E3, 7.00 min; 4-OH-E2, 13.50 min; 2-OH-E2, 15.50 min; 2-OH-E1 and 4-OH-E1 co-eluted at 17.75 min; E2, 24.00 min; E1, 29.50 min. Radiolabeled metabolites were detected and quantified using a Packard Flo-One Beta detector with Ultima Flo M liquid scintillant. The limit of detection for individual metabolite peaks was ~0.3% of total substrate. Tabulated data represent means ± SD from analysis of triplicate incubations.

Western immunoblot

Liver microsomes (50 µg) were resolved by SDS–PAGE on 4–12% gradient NuPage gels in MOPS buffer. They were then transferred to a PVDF membrane (Immobilon-P; Millipore). Non-specific binding was blocked using Tris-buffered saline (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, containing 10% non-fat milk) for 1 h at room temperature. The membranes were probed for 2 h at room temperature with polyclonal antibodies for CYP 1B1, 2B1/2 and 3A1/2. Antibody binding for CYPs 2B1/2 and 3A1/2 was detected using alkaline phosphatase-conjugated anti-rabbit IgG, then visualized using BCIP/NBT phosphatase substrate. Anti-CYP 1B1 antibody was detected by incubation for 1 h with a horseradish peroxidase-linked donkey anti-rabbit IgG using the enhanced chemiluminescence method.

Results

Initial studies of the protein and time dependence of E2 metabolism by RLM from female ACI and Sprague–Dawley rats demonstrated a linear increase from 0.3 to 3.0 mg protein/ml and similar increases from 20 to 60 min incubation (data not shown). Based on these results, the experimental conditions were standardized to 1.0 mg protein/ml and 20 min incubation. Next, microsomes from ACI and Sprague–Dawley rats were incubated with a range of concentrations of E2 from 9 nM to 30 µM, to characterize the dependence on substrate concentration. Striking quantitative and qualitative changes in metabolic profiles were observed as the E2 concentration was decreased.
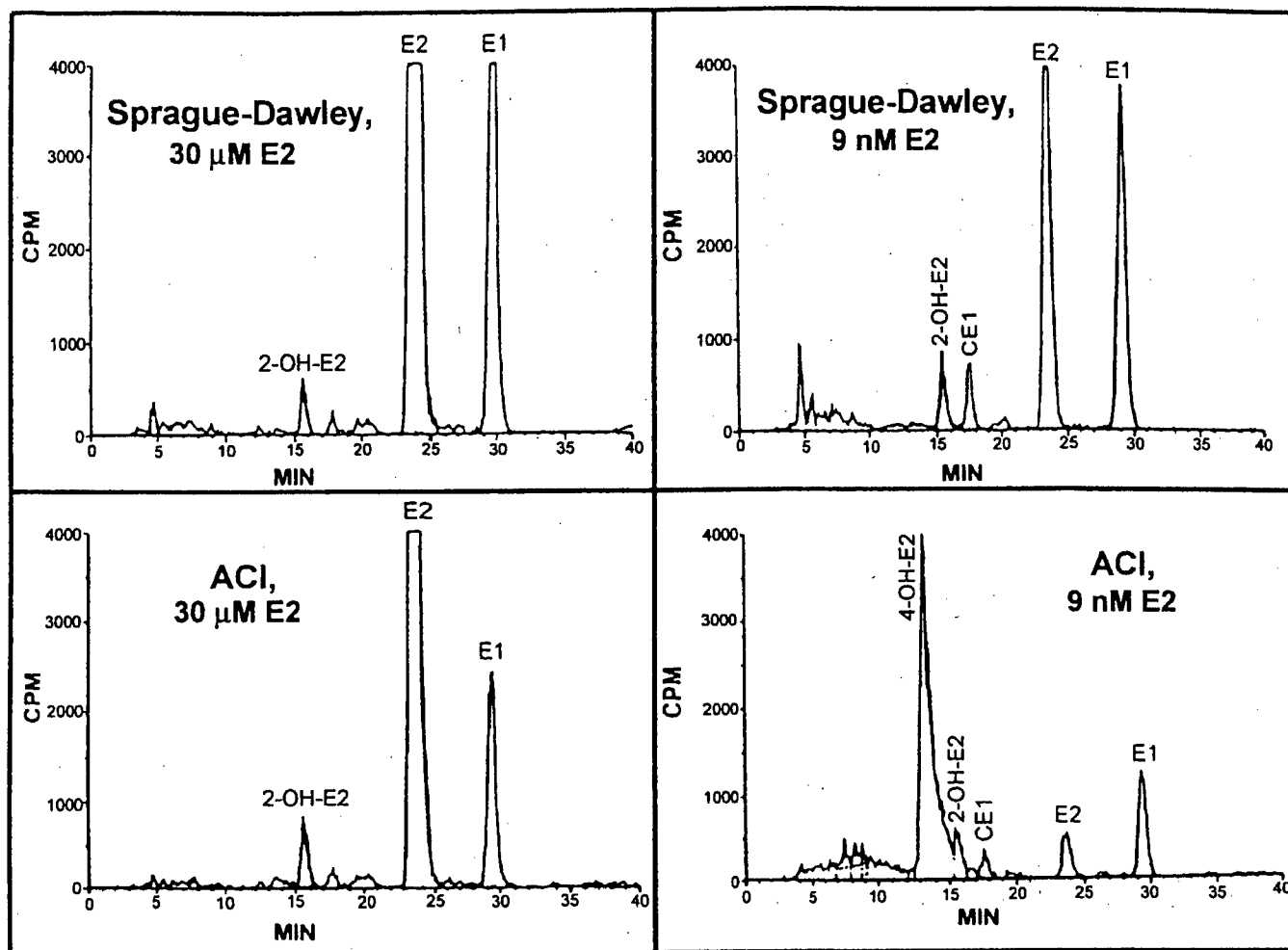
Fig. 1. Estradiol metabolite profiles as a function of strain and estradiol concentration. [^{3}H]E2 was incubated with rat liver microsomes (1 mg microsomal protein/ml) for 20 min with a NADPH generating system. Ethyl acetate extracts were processed and analyzed by reverse phase HPLC as described in Materials and methods. These data were generated using RLM from mature rats. Similar results were obtained with immature animals.

These changes are demonstrated by the data in Figure 1, showing representative HPLC profiles resulting from E2 metabolism at the high and low ends of this concentration range.

With an initial concentration of 30 µM E2 the major metabolite produced by RLM from both strains was E1, with minor amounts of 2-OH-E2 as the only other detectable product (Figure 1A and C). In the Sprague–Dawley preparation containing 9 nM E2 the major metabolite formed was still E1, but there was a nearly equal contribution of aromatic hydroxylation, specifically formation of 2-OH-E2 and 2/4-hydroxyestrone (CE1) (Figure 1B). A more dramatic change in metabolism was seen in the ACI RLM at nanomolar E2 concentrations (Figure 1D). At this E2 concentration E1 was no longer the major metabolite produced and instead aromatic hydroxylation was the major pathway. Moreover, the predominant catechol estrogen formed was 4-OH-E2, with lesser amounts of 2-OH-E2 and CE1 found. Examination of the kinetics of individual product formation at times from 5 to 20 min incubation showed that 2-OH-E2, 4-OH-E2 and E1 yields increased steadily with incubation time, whereas CE1 formation was only detectable after the 5 min incubation time (data not shown). This is consistent with their formation as primary and secondary metabolites of E2. Parallel incubations were performed with ^{3}H- and ^{14}C-labeled E2 at initial substrate concentrations of 0.3, 1 and 10 µM, which produced identical

product distributions, demonstrating that loss of tritium was not a significant factor in our studies (data not shown).

This concentration-dependent shift in the spectrum of metabolites formed by RLM from each strain is shown by the tabulated product yields (Table I) and by the normalized product distributions (Figure 2). The E2 concentrations chosen for this experiment were 30 µM, a concentration where E1 formation predominates, and 100 nM, where both strains produced significant yields of both dehydrogenation and hydroxylation products. At 30 µM E2 the major metabolite produced for both strains and both age groups was E1, accounting for 55–95% of metabolites (Figure 2A). 2-OH-E2 is the next most abundant metabolite, ranging from 8% in immature ACI rats to >40% in mature Sprague–Dawley rats. The only group that produced detectable 4-OH-E2 at this E2 concentration was the mature ACI rats and this was <10% of total metabolites. At 100 nM E2, E1 remained the major product from Sprague–Dawley microsomes, but fell to 45% of total metabolites (Figure 2B). 2-OH-E2 production remained the same in each strain for each age when the E2 concentration was decreased. Also, at 100 nM E2 there was production of CE1 by all groups but immature ACI rats. Formation of the catechol estrones requires two oxidative steps, presumably the conversion of E2 to estrone followed by aromatic hydroxylation to the catechol. Our HPLC procedure could not resolve

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Table I. Effects of strain and substrate concentration on estradiol product yields

Strain	Age (weeks)	[E2]	4-OH-E2 (pmol/mg/min)	2-OH-E2 (pmol/mg/min)	CE1 (pmol/mg/min)	E1 (pmol/mg/min)
Sprague–Dawley	7	100 nM	0.10 ± 0.18	0.79 ± 0.07	0.39 ± 0.19	1.19 ± 0.26
	14	100 nM	<0.015	0.94 ± 0.07	0.60 ± 0.14	1.26 ± 0.04
ACI	7	100 nM	2.79 ± 0.15	0.19 ± 0.17	<0.015	0.97 ± 0.11
	14	100 nM	1.66 ± 0.18	1.32 ± 0.09	0.72 ± 0.10	0.26 ± 0.04
Sprague–Dawley	7	30 μM	<4.5	28 ± 24	<4.5	82 ± 6
	14	30 μM	<4.5	55 ± 6	<4.5	75 ± 26
ACI	7	30 μM	<4.5	12 ± 16	<4.5	251 ± 19
	14	30 μM	51 ± 8	107 ± 14	20 ± 14	428 ± 25

Rat liver microsomes (1 mg/ml) were incubated for 20 min with the indicated concentrations of [^{3}H]E2 with a NADPH generating system. All incubations were performed in triplicate. Extraction and HPLC analysis were as described under Materials and methods. Limits of detection for metabolites were 0.015 pmol/mg/min for 100 nM initial E2 concentration and 4.5 pmol/mg/min for 30 μM initial substrate concentration.
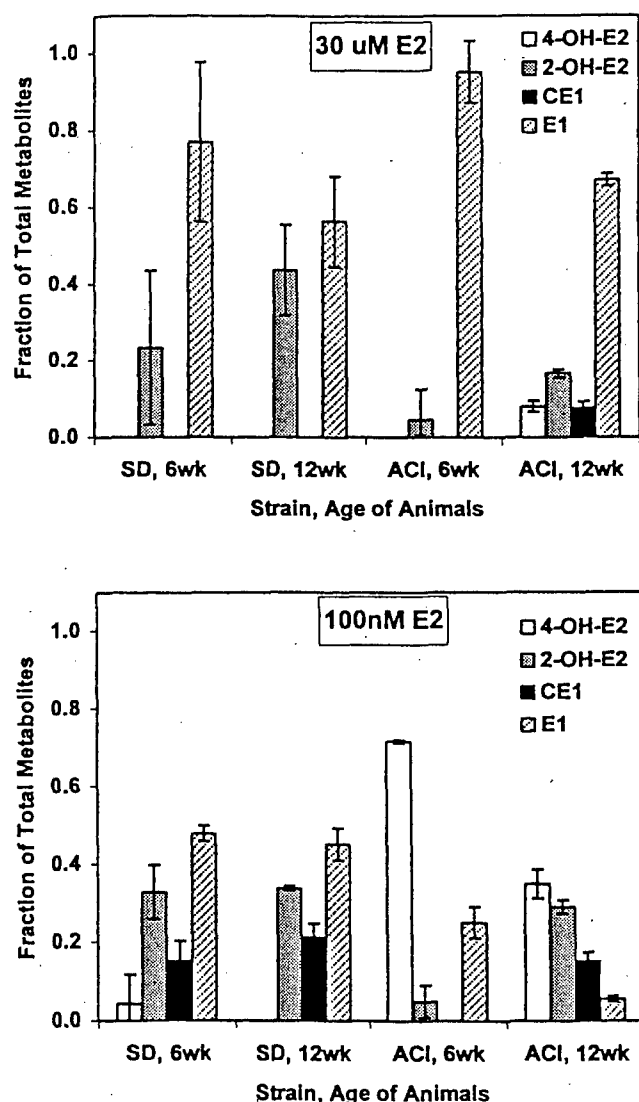

Fig. 2. Product distribution of estradiol metabolites from SD and ACI rat liver microsomes as a function of age and substrate concentration. The total metabolism for each condition, from Table I, was normalized to 1.0. Each incubation contained 1 mg/ml microsomal protein and was incubated with E2 for 20 min with a NADPH generating system. Extraction and analysis were as described under Materials and methods and for Figure 1. Data represent the means ± standard deviations from the analysis of three replicate incubations. Microsomes from immature (7-week-old) and mature (14-week-old) animals were examined in parallel incubations.
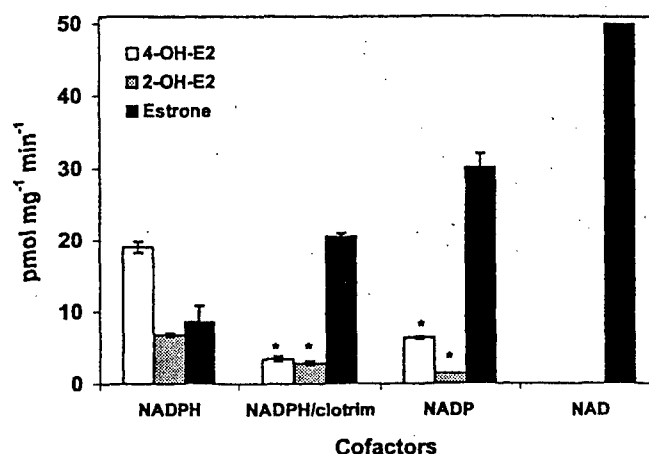

Fig. 3. Modulation of estradiol oxidation by ACI rat liver microsomes. RLM (1 mg/ml) were incubated for 20 min with 1 μM [^{3}H]E2 with either a NADPH generating system or initiation by addition of 1 mM NAD or NADP. Clotrimazole was added 10 min prior to the initiation of reactions. Extraction and analysis were as described under Materials and methods and for Figure 1. Data represent the means ± standard deviations from the analysis of three replicate incubations. Comparison of the rate of individual metabolite formation to the rate observed with the NADPH generating system was performed by one-way ANOVA followed by Dunnett's test. *, Significant difference from NADPH generating system, $P < 0.05$.

the catechol estrones 2-OH-E1 and 4-OH-E1, but it could distinguish the catechol estrones from the catechol estradiols 2-OH-E2 and 4-OH-E2. Strikingly, at 100 nM E2 RLM from both age groups of ACI rats produced more 4-OH-E2 than any other metabolite, accounting for 35% of metabolites in mature ACI rats and 70% of metabolites formed in immature ACI rats. It is key to note that the clear qualitative differences in metabolite profile for E2 oxidation are between strains and that only minor quantitative differences are observed between immature and mature female rats of the same strain.

The ACI preparations were incubated with various cofactors and inhibitors to determine what enzymes or families of enzymes were responsible for E2 metabolism (Figure 3). We chose 1 μM E2 as the initial substrate concentration to ensure significant production of E1 and of both aromatic hydroxylation products. Incubations containing the standard NADPH generating system were used as a control. The major metabolite produced by ACI liver microsomes was 4-OH-E2, with lesser amounts of 2-OH-E2 and E1 being formed. Incubations of 1 μM E2 with ACI RLM with the NADPH generating system and 10 μM clotrimazole, a broad CYP inhibitor, showed a

Table II. Kinetic constants for microsomal estradiol oxidation

Metabolite	Sprague–Dawley		ACI	
	K_m (µM)	V_{max} (pmol/mg/min)	K_m (µM)	V_{max} (pmol/mg/min)
4-Hydroxy-E2	ND	ND	0.8 ± 0.3	19.0 ± 2.0
2-Hydroxy-E2	3.3 ± 1.3	5.8 ± 1.8	4.2 ± 0.8	34.0 ± 2.0
E1	14.8 ± 0.8	386 ± 19	38.0 ± 2.0	414 ± 12

Rat liver microsomes (1 mg/ml) were incubated for 20 min with concentrations of [^{3}H]E2 at half-log intervals from 9 nM to 30 µM with a NADPH generating system. All incubations were performed in triplicate. Extraction and HPLC analyses were as described under Materials and methods. Kinetic constants were derived from the data using the kinetics analysis program GraFit v.4.0. ND, not detected. Limit of detection for individual products was 0.3% of the initial E2 concentration and thus ranged from 0.0014 pmol/mg/min at 9 nM substrate to 4.5 pmol/mg/min at 30 µM E2.

75% decrease in production of 4-OH-E2 and 2-OH-E2, indicating that these are products of CYP-dependent reactions. Also, there was an increase in E1 production, a 17β-hydroxysteroid dehydrogenase (17β-HSD)-dependent reaction. When the cofactor NADP was added without glucose 6-phosphate dehydrogenase there was a 70% decrease in production of 4-OH-E2 and 2-OH-E2, with a corresponding increase in E1 production. This is consistent with the ability of NADP to serve as a cofactor for 17β-HSD (30,31) but not for CYP-dependent hydroxylation. There was some production of 4-OH-E2 and 2-OH-E2, which could be attributed to reduction of the NADP to NADPH by 17β-HSD or other microsomal dehydrogenases, which could then serve as a cofactor for CYP-dependent metabolism of E2 to the catechols. When NAD, a cofactor that cannot be reduced to NADPH, was added there was complete abolition of formation of the catechol estrogens.

The differences in hydroxylation products formed in the two strains of rats suggests that there are different CYPs present in liver microsomes from these two strains. Different enzymes might be expected to exhibit different kinetic constants. Sprague–Dawley and ACI RLM were incubated for 20 min with E2 concentrations of 0.003, 0.01, 0.03, 0.10, 0.30, 1, 3, 10, 30 and 60 µM. Using graphical analysis of initial rate data, kinetic constants were obtained for the three metabolites of interest for ACI and Sprague–Dawley rats (Table II). Catechol estrones, which result from two sequential enzymatic reactions, were not included in the calculation of initial rate, either as distinct products or as contributors to either aromatic hydroxylation of dehydrogenation. We found that the V_{max} and K_m for E1 in both rat strains are significantly higher than for aromatic hydroxylation. This is consistent with the known properties of 17β-HSD, a high capacity and relatively high K_m enzyme for E2 oxidation. Aromatic hydroxylation, however, is catalyzed by CYPs. Second, there is no significant difference between the E2 K_m value for 2-hydroxylation in Sprague–Dawley and ACI rats. In contrast, the E2 K_m value for 4-hydroxylation in ACI rats is significantly lower than the K_m for 2-hydroxylation in ACI rats. These data suggest that the same CYP may catalyze 2-hydroxylation of E2 in both strains, but that the 4-hydroxylation seen with ACI RLM is catalyzed by a different enzyme.

The search for this different CYP-dependent enzyme was carried out by western analysis of RLM. Immunoblot analysis
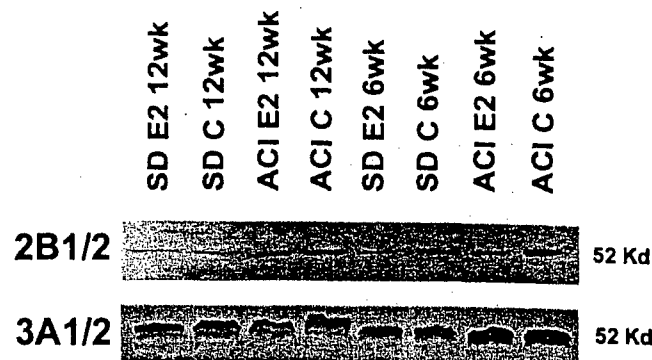

Fig. 4. Western analysis of cytochrome P450 expression in rat liver microsomes. Rat liver microsomes (50 µg) were resolved by SDS–PAGE on 4–12% gradient gels, transferred to a PVDF membrane and probed with polyclonal antibodies for CYPs 2B1/2 and 3A1/2 for 2 h at room temperature. Antibody reactions were detected using alkaline phosphatase-conjugated anti-rabbit IgG, then visualized using BCIP/NBT phosphatase substrate. SD, Sprague–Dawley; C, control; E2, 17β-estradiol-treated; 6 wk, sexually immature animals; 12 wk, sexually mature animals.

was performed on the microsomal fractions of each rat strain and each age group to determine the expression level of CYPs known to be involved in E2 metabolism. Under the conditions studied there is expression of CYPs 2B1/2 and 3A1/2 in both strains of rat (Figure 4). There are no major differences in the expression of CYP 2B1/2 or 3A1/2 between the two age groups of ACI rats. Both immature and mature Sprague–Dawley rats express comparable levels of CYP 2B1/2, but immature Sprague–Dawley rats have diminished 3A1/2 expression relative to mature rats. In this study we also examined the effect of chronic E2 administration on hepatic CYP expression. No effects were detected. This was mirrored by a lack of detectable effect of E2 treatment on the ability of RLM to metabolize E2 (data not shown). ACI and Sprague–Dawley RLM were analyzed for expression of CYP 1B1 as well. There was no detectable expression of CYP 1B1, a known estradiol 4-hydroxylase, in RLM from either strain of rat (data not shown).

Discussion

We have demonstrated that liver microsomes from ACI and Sprague–Dawley rats both efficiently metabolize E2 over a wide range of initial substrate concentrations. These liver preparations catalyze only aromatic hydroxylation and the 17β-dehydrogenation of E2. This is in marked contrast to male Sprague–Dawley liver microsomes, which yield a far more complex mixture of oxidized metabolites (G.A.Reed, unpublished observation; 32). Similar metabolite profiles are produced by RLM from females of the two strains at high initial E2 concentrations (i.e. ≥10 µM), but this changes dramatically when the substrate concentration is lowered into the nanomolar range. Sprague–Dawley rats yield more 2-OH-E2 relative to E1 as the initial substrate concentration is decreased, but E1 remains the major product. In ACI rats, however, aromatic hydroxylation dominates and 4-OH-E2 is produced as the predominant metabolite at lower E2 concentrations.

Previous reports on the metabolism of E2 by microsomal preparations and by purified CYPs have employed extremely high substrate concentrations, ranging from 20 to 200 µM (32–34; reviewed in ref. 35). Our qualitative findings at high E2 concentrations, i.e. that E1 is the major metabolite formed followed by 2-OH-E2, are consistent with other laboratories

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studying E2 metabolism by RLM from female Sprague–Dawley rats and from other strains as well (33,34). With microsomes from the ACI rat, however, this qualitative result changes dramatically, but only when the initial E2 concentration is lowered toward more realistic levels. We have shown that rat liver microsomes oxidize E2 at low concentrations, nearing actual physiological concentrations. These lower, more physiologically relevant concentrations, which have not been examined before, were required in order to observe the dramatic differences in metabolite profiles between these two rat strains.

In rats hepatic hydroxylation of E2 to the catechols 2-OH-E2 and 4-OH-E2 is catalyzed by CYPs 1A2, 2B1/2, 2C6, 2C11, the 2D family and the 3A family (reviewed in 11,35). Production of 2-OH-E2 by these CYPs greatly exceeds formation of 4-OH-E2, with 80–90% production of 2-OH-E2 and only 10–20% production of 4-OH-E2. This greater production of 2-OH-E2 reflects the orientation of E2 in the active site of the enzyme. Thus, formation of more 2-OH-E2 than 4-OH-E2 is a consequence of the binding interaction between substrate and CYP and should not be dependent on the concentration of E2. In contrast, human CYP 1B1 is unique in that it is reported to be a specific 4-hydroxylase of E2 (36). The presence of CYP 1B1 as a constitutive enzyme in female ACI rat liver would be consistent with our observed metabolite profile. By comparing the K_m values determined for ACI RLM, it is apparent that 4-hydroxylation of E2 is catalyzed by a different enzyme with a lower K_m than that for 2-hydroxylation. The K_m determined for 4-hydroxylation of E2 in ACI RLM, substantially lower than that determined for 2-hydroxylation of E2 in the same incubations, matches the reported E2 K_m value for 4-hydroxylation by recombinant human CYP 1B1 (36), providing an additional suggestion that this CYP may be responsible for the observed biotransformation of E2.

We found, however, that in female ACI rat liver there are no qualitative differences in the CYPs tested from what is expressed in the Sprague–Dawley female liver. There was expression of CYPs 2B1/2 and 3A1/2 but no detectable expression of CYP 1B1 in microsomes from either strain of rat. This suggests that 4-OH-E2 formation in ACI rats is due either to an additional CYP not probed for or a polymorphism in an existing CYP that alters the site of E2 hydroxylation. This latter proposal is particularly interesting in the light of a recent report from Shimada et al. (37). They characterized polymorphic human CYP 1B1 variants and found that these variants altered the ratio of 4-hydroxylation to 2-hydroxylation of E2 catalyzed by these enzymes. This subtle modulation of the orientation of E2 in the active site of a CYP and the resultant alteration in the site selectivity for E2 hydroxylation is precisely what we believe is responsible for the different metabolite distribution produced by ACI as opposed to Sprague–Dawley liver.

The proposed mechanisms for the carcinogenic action of estrogens depend on either estrogenicity or reactivity of the active species and both can be modulated by oxidative metabolism of estrogens. 2-OH-E2 has a lower affinity for the estrogen receptor (38,39) and has lower estrogenicity than the parent hormone E2 (40,41). Rapid O-methylation of 2-OH-E2 by catechol O-methyltransferase (COMT) results in a more rapid clearance in vivo and may also result in product inhibition of tumor cell proliferation (42,43). The resulting O-methylation of 2-OH-E2 may be the reason for its lack of carcinogenicity. 4-OH-E2, however, is a carcinogenic metabolite of E2 (44,45). It binds to the estrogen receptor with a similar affinity to E2

and it also activates the estrogen receptor (38). 4-OH-E2 takes longer to dissociate from the estrogen receptor compared with E2, therefore, there could be increased biological effects of 4-OH-E2 in comparison with E2 (46). COMT-catalyzed O-methylation is inhibited by 4-OH-E2. Regardless of the mechanism, 4-OH-E2 is important in the carcinogenicity of E2. It is not surprising that ACI rats, which are more sensitive to E2 as a mammary carcinogen, produce more of this detrimental metabolite 4-OH-E2 than Sprague–Dawley rats at near physiological levels of substrate.

Liver metabolism of E2 is an important first step in understanding the role of metabolism in mammary carcinogenesis. The liver plays a role in controlling the systemic levels of circulating metabolites. In addition to the liver, however, there is much interest in the metabolism of E2 in target tissues (35). Our next goal is to investigate the metabolism of E2 in the mammary gland to see whether there are differences in the target tissue that may be responsible for the differences in susceptibility of the two strains of rat. If there are differences in metabolism in the target tissue (the mammary gland) this would provide additional support for the role of metabolism of E2 in carcinogenicity.

In summary, these results reveal that there are dramatic differences in the hepatic metabolism of E2 in ACI and Sprague–Dawley rats and that these differences are only apparent as the conditions approach physiological concentrations of E2. Since 4-OH-E2 is thought to be the main carcinogenic metabolite of E2, it is even more interesting that ACI rat liver produces primarily this metabolite when challenged with near physiological levels of E2. The exclusive formation of this metabolite by the rat strain which is more sensitive to the carcinogenic effects of E2 suggests a role for this difference in E2 disposition in the different responses of the strains.

Acknowledgements

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